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PRINCIPAL INVESTIGATOR: **PI: Jeffrey Alan Plunkett, Ph.D.**
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14. ABSTRACT We hypothesize that the ability to grow an axon over CSPGs is intrinsic to adult zebrafish brainstem neurons and entails the expression of a distinct set of genes. This premise will be addressed using *in vitro* adult zebrafish brainstem cell culture systems and *in vivo* adult zebrafish spinal cord injury model systems. In cultures we have observed three distinct populations of brainstem neurons with regard to their response to chondroitin sulfates (CS). Some cells attach, extend processes, and remain exclusively associated with CS. Other cells attach outside and extend processes into CS-rich areas. A third kind of cell was found to attach outside and extend processes up to but never into CS-rich areas. In fact, these processes were clearly repelled by CS. We are currently quantifying different aspects of these three adult zebrafish brainstem neuron populations. It is clear from our first data that we are able to isolate adult zebrafish brainstem neurons and maintain these in culture in the presence of CS, while typical characteristics in relationship to CS remain present. Thus these cultures mimic the in vivo behaviors of brainstem populations after SCI. In parallel to these in vitro studies, we have developed minimally invasive spinal cord transection and tracer injection techniques. These are currently employed to investigate the evolution of the scar and the time course of axon regeneration after spinal cord injury. The data from these first in vivo experiments will serve as a basis to optimize our harvest of retrogradely labeled adult brainstem neurons that did or did not regenerate their axon beyond a transection site.

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Contract # W81XWH-09-1-0403

Title: Molecular Determinants Fundamental to Axon Regeneration after SCI

PI: Dr. Jeffrey Alan Plunkett

Scientific Progress from June 9, 2009 – June 9, 2010 (Months 1-12)

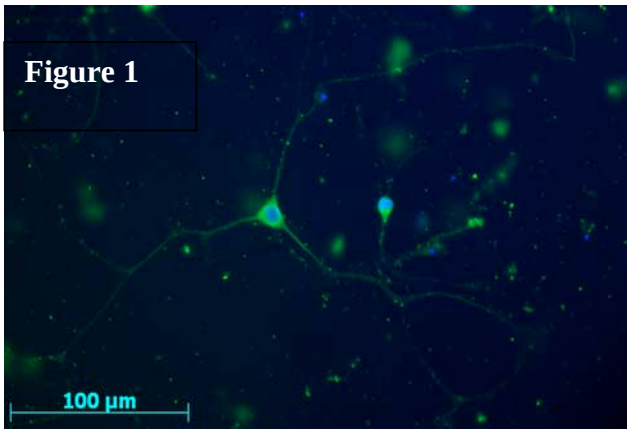
Introduction:
The zebrafish spinal cord model system is unique because of the co-existence of brainstem neurons that do (regenerators) and others that don't (non-regenerators) grow their axon beyond a spinal cord injury. These responses occur in the presence of CS-PGs, which are well-known inhibitors of axon growth in the injured mammalian spinal cord. In this proposal (the first phase of a long-term plan), we will use an *in vitro* and an *in vivo* model system to address the overall hypothesis that *the axon growth response in the injured zebrafish spinal cord is intrinsic to brainstem neurons and entails the expression of a distinct set of genes.* In Specific Aim 1, we will determine *in vitro* the effect of growth-inhibitory CSs on axon growth from primary brainstem neurons from the adult zebrafish. The experiments in Specific Aim 2 are designed to reveal the involvement of L1.1 in axon growth. In Specific Aim 3, we will identify genes that are fundamental to successful axon regeneration past a CS-PG-rich area in the injured spinal cord.

Body:

SOW:
<u>Specific Aim 1:</u> To determine *in vitro* the effect of growth-inhibitory chondroitin sulfates on axon growth from adult zebrafish primary brainstem neurons.
Experiments will be performed in established culture model systems to determine the extent of axon growth by isolated zebrafish brainstem neurons grown on substrates of purified CS. Zebrafish brainstem neurons have been cultured in the Plunkett laboratory in preliminary experiments. The dishes will be covered entirely with CS. Control cultures will have no CS (Months 1-9).

Milestones:
Specific Aim 1. Postdoctoral Fellow/Research Technician (tbd); Dr. Plunkett's laboratory.
Month 1-3: Establish neuronal cultures, including different coatings.

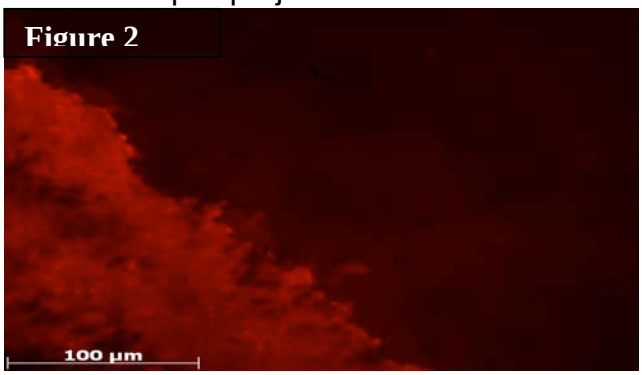
The ACURO approval for animal use was reviewed and approval was granted for work on specific aims 1 and 2 on August 24, 2009. A Postdoctoral Fellow was hired at St. Thomas



University to carry out the work set forth by Aims 1 and 2. The Postdoctoral researcher is Dr. Alexis Tapanes-Castillo who had her training in the laboratory of Prof. Vance Lemmon at the Miami Project to Cure Paralysis, University of Miami, Miami, FL.

Culture conditions for the establishment of adult brainstem cultures have been accomplished in the 3 month review period. Specific concentrations for plate coating with nitrocellulose, Poly-D lysine and laminin have been established. In addition, plating densities of brainstem cells per well have been established. We are also currently working on serum-free and serum weaning procedures for the brainstem cells that will allow for an environment free of growth-permissive cues, which could interfere with our analyses. With culture techniques previously established we have also found a technique using fluorescent antibodies to label axons and brainstem cells. Using antibodies directed against tubulin we have found that under appropriate culture conditions we now can visualize cells with > 100um processes after 6 days in culture and have determined this time-frame to be appropriate to carry out the experiments. Furthermore, the fluorescent tubulin labeling has allowed us to visualize many more cells than detected previously using DIC light microscopy. A micrograph of cells labeled with antibodies against tubulin is shown in **Figure 1**.

In other experiments we have also established proper CS coating techniques on substrates described above. As shown in **Figure 2**, we are able to mix CS with rhodamine dextran and so exactly detect the location of CSs on the culture dish substrate (note: in figure 2, CS is fluorescent red). We will be using this procedure to coat the entire well to establish the ability of brainstem cells to grow on this CS-containing substrate. We are also working on the establishment of control cultures for our CS substrate experiments. These controls involve the use of optic projection neurons in which a pre-conditioning optic crush has been performed. Pieces of retina are attached to substrates containing CS and as Becker and colleagues demonstrated (J. Neurosci. 22(3):842-853, 2002) the regenerating optic projection axons will avoid areas of active CS. This control will determine in our experiments if the CSPG substrates applied to the surface are actively inhibiting axon growth. We currently have performed the optic crush experiments to pre-lesion the optic projection axons and plated the retina tissues 7 days following the crush. Results thus far in several wells examined have indicated that our substrates appear to be inhibitory to the optic projection axons. However, more experimentation is currently ongoing to verify these results.



Month 4-6: Test CS inhibitory action and loss after treatment with chondroitinase ABC.
Month 7-9: Perform Exp. #1 and #2 (all conditions simultaneously).

Culture conditions for the establishment of adult brainstem cultures have been accomplished in the 3 month review period. Specific concentrations for plate coating with nitrocellulose, Poly-D lysine and laminin have been established. In addition, plating densities of brainstem cells per well have been established. We have also established serum-free and serum weaning procedures for the brainstem cells that will allow for an environment free of growth-permissive cues. In separate experiments we have also established proper CS coating techniques on substrates described above.

In cultures described above we have observed three distinct populations of cells with regard to their response to CS presented to them in culture. We have identified populations of cells that attach, extend processes and remain exclusively associated within the CS area on the plate. We have also observed a population of cells that attach outside and extend processes into the CS rich areas. Finally, we have observed a population of cells that attach outside, extend processes towards but never into CS areas. These processes are repelled by the CS. We are currently performing experiments in which we have chondroitinase ABC treated the substrates. However, because we currently have populations of cells that grow inside, and cross the CS barrier we feel that chondroitinase ABC treatment experiments will yield little informative data. Because we also have cells that cross into and are repelled by CS barriers in the same culture dish, the optic crush control experiments will not be necessary for future experiments. The brainstem neurons that are inhibited by the CS border serves as its own control for CS border inhibitory efficacy.

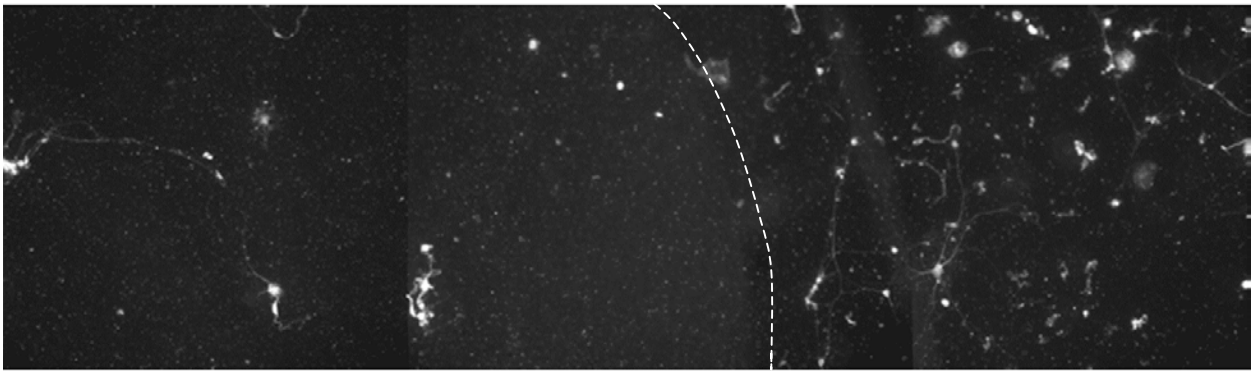


Figure 3. Montage image of cultured brainstem cellular responses to CS. Figure shows two of the cellular responses to CS. Dashed line represents the CS border. The left side of the dashed line is coated with CS and the right side of the line is absent of CS. Arrows on left side of figure show a cell extending processes in an area of CS. Arrows on the right side of figure show cells extending processes in an area devoid of CS, and avoiding the CS border (dashed line).

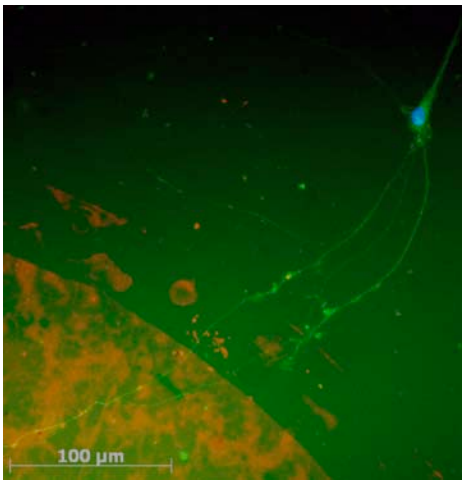


Figure 4. Triple-label immunofluorescent images of cultured brainstem cells extending processes and entering areas of CS. CS are labeled with rhodamine dextran (red) and axonal processes are labeled with anti-tubulin antibodies with a fluorescein (green) secondary. Cell nuclei are labeled with DAPI (blue). Figure clearly shows a population of adult brainstem neurons that cross into areas of CS. Note of added proof: within the same culture well, we observed cells that extended processes and avoided the CS border. This indicates that our CS border was truly inhibitory to other populations of cells with in the same culture well.

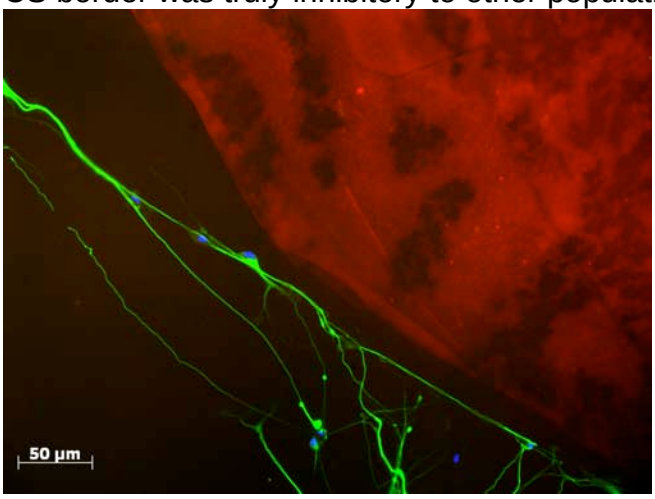


Figure 5. Double-label immunofluorescent image of cultured brainstem cells extending processes and avoiding areas of CSs. CSs are labeled with rhodamine dextran (red) and axonal processes are labeled with anti-tubulin antibodies with a fluorescein (green) secondary. Figure clearly shows a population of adult brainstem neurons that avoid areas of CS.

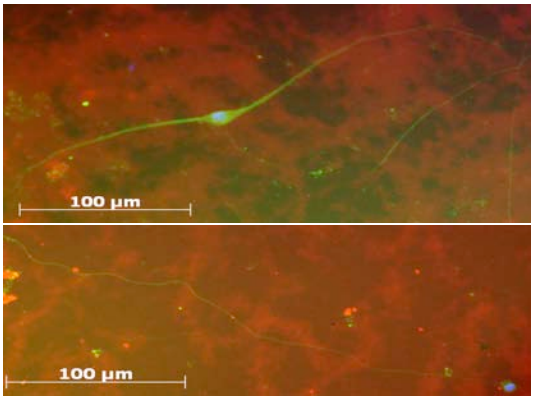


Figure 6. Triple-label immunofluorescent images of cultured brainstem cells extending processes in areas of CS. CS are labeled with rhodamine dextran (red) and axonal processes are labeled with anti-tubulin antibodies with a fluorescein (green) secondary. Cell nuclei are labeled with DAPI (blue). Figure clearly shows a population of adult brainstem neurons capable of extending processes within CS. Note of added proof: within the same culture well, we observed cells that extended processes and avoided the CS border. This indicates that our CS border was truly inhibitory to other populations of cells with in the same culture well.

Month 10-12: Analyze results Exp. #1 and #2. Perform Exp. #3.

Culture conditions for adult brainstem cultures have been further refined in the 3 month review period. We have established serum-free and serum weaning procedures for the brainstem cells that will allow for an environment free of growth-permissive cues. In cultures described above we have observed three distinct populations of cells with regard to their response to CS presented to them in culture. We are currently performing experiments in which we have quantitatively analyzed neuronal populations and their interactions with CS borders. Laminin-coated plates received spots of CS. We have identified populations of cells that attach and extend processes that remain exclusively associated with CS **(Figures 6 and 7)**. These cells with neurites appeared to prefer substrates of CSPG. We have also observed a population of cells that attach outside and extend processes into CS-rich areas **(Figures 4 and 7).** Finally, we have observed a population of cells that attach outside the CS area and extend processes up to the CS border but appear to be repelled by the CS **(Figures 5 and 7)**.

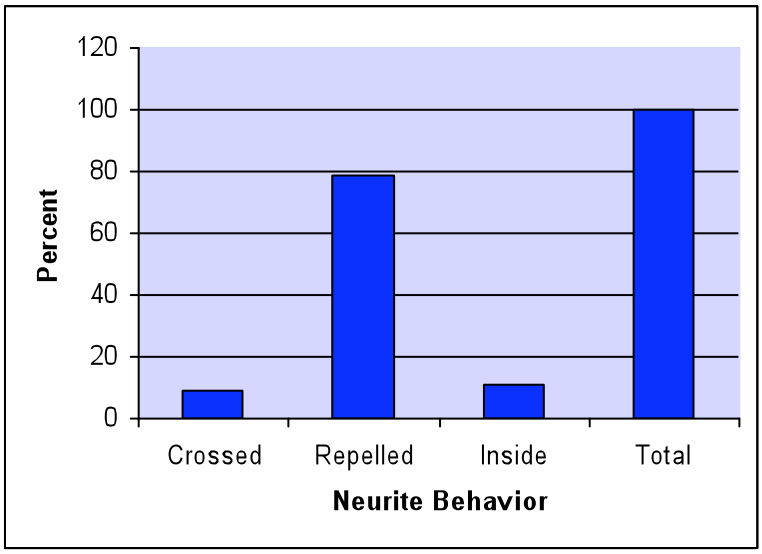


Figure 7. Cultured brainstem cellular responses to CS. Quantitative data were obtained from three independent experiments. In total 99 cells were included in the analysis. All analyzed cells were within 20 μM of a CS border. Graph demonstrates that of the total number of neurites analyzed, 9% of the neurites crossed from areas of laminin alone into CS containing areas (*Crossed)*. Within the same cultures, 79% of the neurites were repelled at CS borders *(Repelled)*. These resulted indicated that CS are inhibitory to a sub-population of brainstem neurons. A population of brainstem neurons was also observed that exclusively associated with CS *(Inside)*.

SOW:

Specific Aim 2: To determine *in vitro* the involvement of L1.1 in axon growth from adult zebrafish primary brainstem neurons over growth-inhibitory chondroitin sulfates. Experiments will be carried out using established culture model systems to determine the extent of involvement of L1.1 in the ability of adult zebrafish brainstem neurons to grow their axon on substrates of chondroitin sulfates. L1.1 levels in cultured neurons will be perturbed using antisense morpholino and lentiviral vectors (to be generated in the Viral Vector Core facility at the University of Pittsburgh). There is ample evidence in the literature from in vivo and in vitro experiments that L1.1 (as well as its homolog in mammals, L1) is crucial for axon regeneration. Thus our data are physiologically relevant for human spinal cord injury/repair (Months 9-18).

Milestones:

Specific Aim 2.

Month 1-6: Prepare/optimize L1.1 antisense/control morpholino and LV-L1.1.

Month 7-12: Test morpholino and LV effects on L1.1 in neurons. Perform Experiments.

To determine the extent of involvement of L1.1 in the ability of adult zebrafish brainstem

neurons to grow their axon on substrates of CS, we will perturb L1.1 levels in cultured neurons using antisense morpholino oligos. Two morpholinos were ordered from Gene Tools, LLC (www.gene-tools.com): (1) a fluoresceinated standard control 5'-CCTCTTACCTCAGTTACAATTTATA-3' and (2) a fluoresceinated morpholino directed against L1.1 5'-ATGAAAACAGCCCCGACTCCAGACA-3', which was reported to significantly reduce L1.1 immunolabeling in vivo (Becker et al., 2004). To deliver morpholinos into cultured neurons, we are currently using Endo-porter (Gene Tools), a weak-base amphiphilic peptide that has been used to efficiently deliver morpholinos into cultured cells (Summerton, 2005). In preliminary experiments, we added the standard control morpholino to 96-hour adult brainstem neuronal cultures and have determined that control morpholino was efficiently delivered into some brainstem cells **(Figure 8)**. Experiments are currently underway to optimize morpholino delivery. We will then test the effect of the L1.1 morpholino on neurons exposed to growth-permissive PDL/laminin (control) and growth-inhibitory CS.

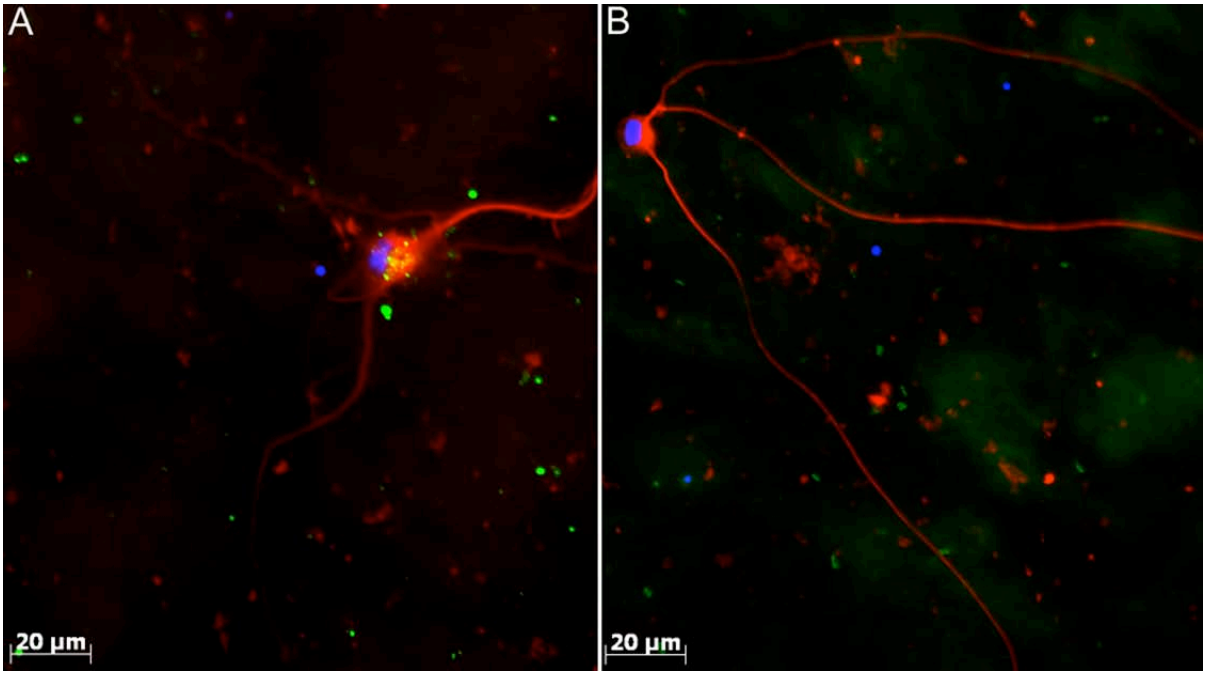


Figure 8. Delivery of control morpholinos into cultured brainstem cells
Neuronal processes are labeled with an anti-tubulin antibody and an Alexa-594 (red) secondary. Cell nuclei are labeled with DAPI (blue). Control morpholino labeled with fluorescein (green) is present in the cell body of the cell **(panel A)**. Panel B shows a cell from the same culture well that did not incorporate morpholino. Our results set the stage for delivery of L1.1 morpholinos.

SOW:

Specifc Aim 3: To identify genes involved in axon regeneration from brainstem neurons in the injured adult zebrafish spinal cord.

Experiments will be performed to label brainstem neurons with fast blue that do or do not, regenerate an axon across an injury in the adult zebrafish spinal cord. Non-regenerators will be collected from the brainstem of zebrafish that received no spinal cord injury or an injury including a piece of Teflon to prevent any regeneration. Laser capture microdissection techniques will be used to collect these two neuron populations. Their mRNA will be isolated and used on zebrafish microarrays to reveal genes that are differentially expressed. We will reference our results to the gene database of the University of Kentucky (http://scigenes.uky.edu) and NCBI "Entrez Gene" (search axonal regeneration) www.ncbi.nlm.nih.gov database to allow us to determine genes important for axon regeneration. We will share out data with the NCBI Gene Expression Omnibus Database and the Allen spinal cord injury atlas if appropriate to allow fellow scientist to profit from the data. The known ability of zebrafish brainstem neurons to grow across CS-rich areas in the injured spinal cord makes them essentially different from rat neurons that are unable to do so. Even conditioned sensory neurons in rat are not able to grow across an established scar with CS present. Thus, the zebrafish spinal cord injury model allows us to study gene expression in neurons that regenerate their axon through an spinal cord injury milieu that has CS present as in mammals but through which mammalian neurons cannot extend their axon (Months 1-18).

Milestones:

Specific Aim 3. Postdoctoral Fellow (tbd); Dr. Oudega's laboratory.

Month 1-6: Gain experience in spinal cord transection and tracing surgeries. Evaluate surgeries using histology. Practice LCM technique.

The ACURO approval for animal use as described in Aim 3 was reviewed and, after some revisions, approved September 23, 2009. A Postdoctoral Associate was hired early September to perform the experiments described in Aim 3. The Postdoctoral researcher is Katarina Vajn who received her MD and PhD degree from the University of Osijek in Croatia. Several surgical approaches were tested with the objective to keep the overall damage minimal. With histological analyses we were able to select the least invasive surgical approach for injuring the spinal cord effectively. We are now well on our way to investigate in detail the evolution of the injury-induced scar tissue. The results from this study will support execution of our time-course experiment for retrograde tracing.

Month 7-12: Start tracing brainstem neuronal subpopulations. Collect cells with LCM. Collect mRNA and prepare for microarrays. Hybridize microarrays (n=3).

We have made a series of spinal cord injuries using different approaches; from less to more invasive. With classic histological techniques we are currently investigating which one of these techniques would be best to use for our experiments. Our criteria to select the best approach

are completeness of the transection and amount of additional tissue loss. Once we have chosen our approach we can start injuring fish and backfill brainstem neurons that have regenerated their axon beyond the injury. We have established a collaboration with a laboratory at Pitt that owns a LCM device. We can freely use this device and have started practicing. In parallel to the above, we have optimized histological steps such as fish perfusion, spinal cord/brain collection, and cryostat and paraffin sectioning. Importantly, we have tested a variety of antibodies for their usefulness to stain zebrafish spinal cord. Many of the widely used CNS antibodies cannot be used for zebrafish. Incubation time and circumstances as well as concentrations have been established for the majority of these antibodies. This allowed us to start two pivotal experiments. One, we will investigate the evolution of the scar tissue that forms at the spinal cord transaction site. Precise information on the scar development will allow us to identify the best time to inject the tracers and correlate our labeling data with CS presence in the scar. Two, we will investigate axon regeneration from brain stem neurons in time after a spinal cord transection. The data from this experiment will allow us, in combination of the previous experiment, to determine the optimal time point for tracer injection.

Microarray analysis. To identify genes involved in axon regeneration from brainstem neurons in the injured adult zebrafish spinal cord, we will analyze the gene expression profile of two neuronal populations: (1) neurons that regenerate across an injury site and (2) neurons that have not undergone regeneration. Neurons will be collected from injured adult fish or control adult fish using laser capture microdissection techniques. Their mRNA will be isolated and then submitted for microarray analysis to identify genes that are differentially expressed. In preparation for these experiments, we have begun optimizing our RNA purification techniques. We have isolated RNA from adult zebrafish brains using Qiagen's RNeasy Plus Mini Kit. We have also performed reverse-transcriptase PCR reactions to assess the quality of our RNA using β-actin primers and the Superscript III First-Strand Synthesis system (Invitrogen). Establishment of these procedures is critical to the development of techniques used in microarray analysis.

Key Research Accomplishments:

(1) Established optimal brainstem culture and immunostaining conditions.

(2) Observed that cultured adult brainstem neurons respond differently to CS.

(3) Characterizing culture system up to 14 days in vitro, including growth in serum-free media.

(4) Began quantifying neuronal response to CS data.

(5) Achieved morpholino delivery into cultured cells.

(6) Established minimally invasive surgical techniques for spinal cord transection.

(7) Optimized all techniques to acquire best immunocytochemical and histological staining.

(8) Tested antibodies necessary to study spinal cord injury and its consequences.

(9) Started to evaluate the evolution of the glial scar after spinal cord transection.

(10) Started to investigate the time-course of axon regeneration beyond a transection in the spinal cord.

(11) Established techniques to retrogradely label brainstem neurons.

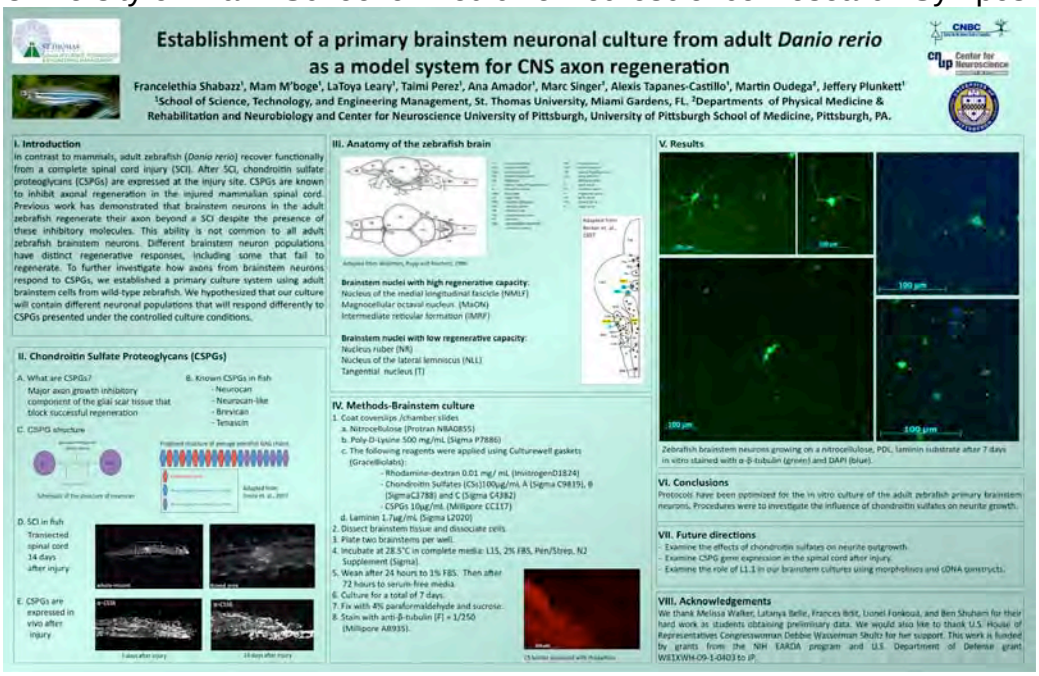
(12) Started to collect labeled neurons for LCM.

(13) Began gene expression studies in zebrafish central nervous system using reverse transcriptase PCR.

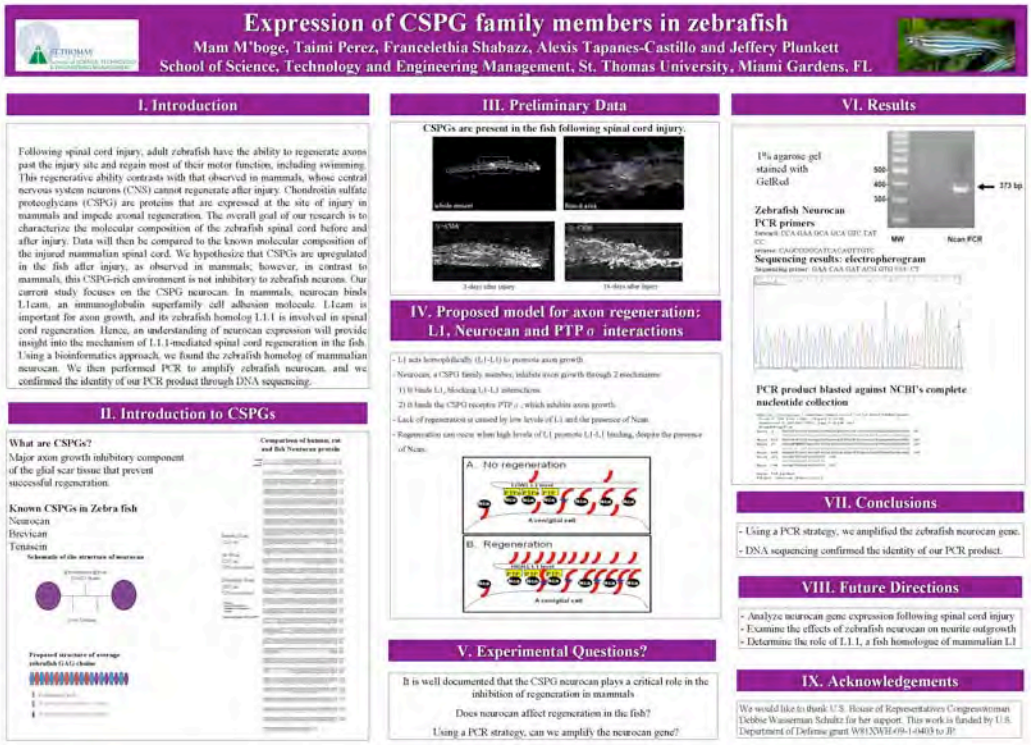
Reportable Outcomes:

Meeting Abstracts:
See Appendix below

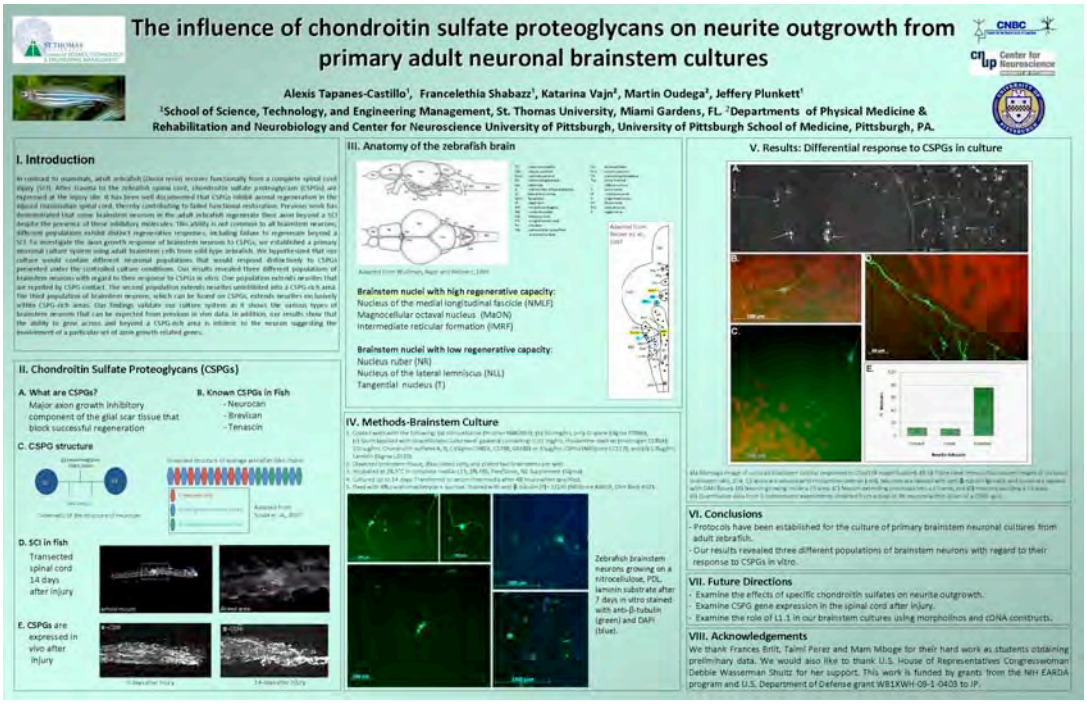
Posters presented:
University of Miami School of Medicine Neuroscience Research Symposium (Nov 2009)



Southeast Florida Cell Science Undergraduate Research Symposium (April 2010)



9th International Meeting on Zebrafish Development and Genetics (June 2010)



Student Success:
- Undergraduate students from St. Thomas University and the Plunkett Lab have or will be presenting their research at the following meetings:

- University of Miami School of Medicine Neuroscience Symposium
- 2nd Annual STEM Undergraduate Conference at Barry University
- Southeast Florida Cell Science Undergraduate Research Symposium
- 9th International Meeting on Zebrafish Development and Genetics (June 2010)
- Society for Neuroscience Meeting, San Diego, CA (Nov. 2010)

This experience has enabled students to gain presentation experience. Undergraduate students have presented posters at 3 meetings in the last 6 months.

- Lionel Fonkoua a May 2009 graduate has been accepted to medical school at Penn State University.

- Frances Brlit a May 2009 graduate has been accepted to the DO program at Nova Southeastern University.

- Taimi Perez a May 2010 graduate has been accepted to the Summer Minority Student MCAT program at the University of Miami Miller School of Medicine.

- Francelethia Shabazz student and technician in the Plunkett Lab presented a talk entitled "The influence of chondroitin sulfate proteoglycans on neurite outgrowth from primary adult neuronal brainstem cultures" at the 5th Annual Southeast Florida Cell Science Undergraduate Research Symposium.

- The first annual Neuroscience Consortium meeting was held January 22, 2010 at St. Thomas University. This meeting brought in consortium members from University of Pittsburgh to discuss progress of the project. Students and post-docs from both institutes benefited from the discussions concerning their particular aspect of the project.

- A manuscript entitled: Establishment and characterization of primary neuronal cultures from adult zebrafish brainstem. Tapanes-Castillo A, Vajn K, Shabazz F, Oudega M and Plunkett JA is in preparation to be submitted by early fall 2010.

Conclusion:

The different studies within this proposal (in vitro as well as in vivo) have been progressing reasonably well according to the described milestones. Some technical/experimental barriers were encountered and these needed to be overcome. This was accomplished for most of them and is still in progress for few. Considering our previous success with surmounting these roadblocks, we are confident that we will be successful. Thus, in conclusion, we are well on our way to accomplish the goals for this first year as they were described in our proposal. This implies that at the end of the 18 month period we will have 1. Analyzed in vitro the different brainstem neuronal populations in the adult zebrafish and their behavior in response to CS; 2. Employed L1.1. morpholinos and analyzed their effects; 3. Analyzed gene expression profiles in neurons that do and those that don't regenerate their axon across a spinal cord transection site. These results will set us up to deeper investigations in the second phase of our proposal.

References:

Becker CG, Becker T (2002) Repellent guidance of regeneration optic axons by chondroitin sulfate glycosaminoglycans in zebrafish. J Neurosci 22(3): 842-853

Plunkett JA, Zambrano A, Fernandez L, Oudega M. (2006) Analysis of chondroitin sulfate proteoglycan expression in the transected zebrafish *Danio rerio* spinal cord. Soc Neurosci 31.

Appendices:

Poster Abstracts

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2009) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
\18th Annual University of Miami Miller School of Medicine Neuroscience Research Day, poster, University of Miami Miller School of Medicine, Miami, FL.

In contrast to mammals, adult zebrafish (*Danio rerio*) can recover from a central nervous system (CNS) injury and resume near normal swimming behavior about three months after a complete spinal cord transection. Strikingly, we found that recovery takes place despite the expression of chondroitin sulfate proteoglycans (CSPGs) at the injury site. CSPGs are known to be expressed in the CNS after injury and prevent axonal regeneration across the glial scar in mammals. However, since zebrafish brainstem neurons have the ability to regenerate axons past the site of injury, our data suggest that unlike mammals, zebrafish can regenerate CNS axons across a CSPG-rich environment. Yet, this ability is not common to all zebrafish brainstem neurons. It has been previously reported that different brainstem neuron populations have distinct regenerative responses: some regenerate after injury, while others do not. To further investigate how brainstem axons respond to CSPGs, we established a primary culture system using adult brainstem cells from wild-type zebrafish. We hypothesize that our culture will consist of different neuronal populations that will respond differently to CSPGs presented under the controlled culture conditions. We expect some neurons will extend axons across CSPG-rich areas, while other neuronal populations will have their axons repelled by CSPGs. Future experiments aim to understand the genetic differences between brainstem neuron populations that exhibit distinct regenerative responses.

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2010) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
2nd Annual STEM Research Symposium, poster, Barry University, Miami Shores, FL.

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury (SCI). After SCI, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. CSPGs are known to inhibit axonal regeneration in the injured mammalian spinal cord. Previous work has demonstrated that brainstem neurons in the adult zebrafish regenerate their axon beyond a SCI despite the presence of these inhibitory molecules. This ability is not common to all adult zebrafish brainstem neurons. Different brainstem neuron populations have distinct regenerative responses, including some that fail to regenerate. To further investigate how axons from brainstem neurons respond to CSPGs, we established a primary culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture will contain different neuronal populations that will respond differently to CSPGs presented under the controlled culture conditions. *Supported by the NIH EARDA program and U.S. Department of Defense grant W81XWH-09-1-0403.*

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2010) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
5th Annual Southeast Florida Cell Science Undergraduate Research Symposium, poster, St. Thomas University, Miami Gardens, FL.

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M'boge, M., Perez, T., Shabazz, F., Tapanes-Castillo, A. and Plunkett, J.A. (2010) Expression of CSPG family members in zebrafish.
5th Annual Southeast Florida Cell Science Undergraduate Research Symposium, poster, St. Thomas University, Miami Gardens, FL.

Following spinal cord injury, adult zebrafish have the ability to regenerate axons past the injury site and regain most of their motor function, including swimming. This regenerative ability contrasts with that observed in mammals, whose central nervous system neurons (CNS) cannot regenerate after injury. Chondroitin sulfate proteoglycans (CSPG) are proteins that are expressed at the site of injury in mammals and impede axonal regeneration. The overall goal of our research is to characterize the molecular composition of the zebrafish spinal cord before and after injury. Data will then be compared to the known molecular composition of the injured mammalian spinal cord. We hypothesize that CSPGs are upregulated in the fish after injury, as observed in mammals; however, in contrast to mammals, this CSPG-rich environment is not inhibitory to zebrafish neurons. Our current study focuses on the CSPG neurocan. In mammals, neurocan binds L1cam, an immunoglobulin superfamily cell adhesion molecule. L1cam is important for axon growth, and its zebrafish homolog L1.1 is involved in spinal cord regeneration. Hence, an understanding of neurocan expression will provide insight into the mechanism of L1.1-mediated spinal cord regeneration in the fish. Using a bioinformatics approach, we found the zebrafish homolog of mammalian neurocan. We then performed PCR to amplify zebrafish neurocan, and we confirmed the identity of our PCR product through DNA sequencing.

Shabazz, F., M'boge, M., Leary, L., Perez, T., Amador, A., Singer, M., Tapanes-Castillo, A., Oudega, M., Plunkett, J.A. (2010) Establishment of a primary brainstem neuronal culture from adult *Danio rerio* as a model system for CNS axon regeneration.
9th Annual Student and Faculty Undergraduate Research Symposium, poster, St. Thomas University, Miami Gardens, FL.

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9th International Meeting on
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WEDNESDAY, JUNE 16 - SUNDAY, JUNE 20, 2010

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The Influence of Chondroitin Sulfate Proteoglycans on Neurite Outgrowth from Primary Adult Neuronal Brainstem Cultures

Alexis Tapanes-Castillo[1], *Francelethia Shabazz*[1], *Katarina Vajn*[2], *Martin Oudega*[2], *Jeffery A. Plunkett*[1]

[1] St. Thomas University, Miami Gardens, FL, USA, [2] University of Pittsburgh, Pittsburgh, PA, USA

In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury (SCI). After trauma to the zebrafish spinal cord, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. It has been well documented that CSPGs inhibit axonal regeneration in the injured mammalian spinal cord, thereby contributing to failed functional restoration. Previous work has demonstrated that some brainstem neurons in the adult zebrafish regenerate their axon beyond a SCI despite the presence of these inhibitory molecules. This ability is not common to all brainstem neurons; different populations exhibit distinct regenerative responses, including failure to regenerate beyond a SCI. To investigate the axon growth response of brainstem neurons to CSPGs, we established a primary neuronal culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs presented under the controlled culture conditions. Our results revealed three different populations of brainstem neurons with regard to their response to CSPGs in vitro. One population extends neurites that are repelled by CSPG contact. The second population extends neurites uninhibited into a CSPG-rich area. The third population of brainstem neurons, which can be found on CSPGs, extends neurites exclusively within CSPG-rich areas. Our findings validate our culture system as it shows the various types of brainstem neurons that can be expected from previous in vivo data. In addition, our results show that the ability to grow across and beyond a CSPG-rich area is intrinsic to the neuron suggesting the involvement of a particular set of axon growth-related genes.

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The following three abstracts were submitted for the upcoming November 2010 SFN meeting:

Control/Tracking Number: 2010-S-6281-SfN
Primary neuronal brainstem culture from adult zebrafish: interactions with an inhibitory chondroitin sulfate proteoglycan-rich environment

AUTHOR BLOCK: A. TAPANES-CASTILLO[1], F. SHABAZZ[1], K. VAJN[2], M. OUDEGA[2], *J. A. PLUNKETT[1];
[1]St Thomas Univ., Miami Gardens, FL; [2]Univ. of Pittsburgh, Pittsburgh, PA
Abstract:
In contrast to mammals, adult zebrafish (*Danio rerio*) recover functionally from a complete spinal cord injury. After trauma to the zebrafish spinal cord, chondroitin sulfate proteoglycans (CSPGs) are expressed at the injury site. It has been well documented that CSPGs inhibit axonal regeneration in the injured mammalian spinal cord, which contributes to the lack of endogenous functional restoration. Previous work in our laboratory has demonstrated that brainstem neurons in the adult zebrafish can regenerate their axon beyond a spinal cord lesion despite the presence of these inhibitory molecules. This ability is not characteristic for all brainstem neurons; different populations exhibit distinct regenerative responses, including failure to regenerate beyond the lesion site. To investigate the axonal growth response of zebrafish brainstem neurons to CSPGs, we developed a primary neuronal culture system using adult brainstem cells from wild-type zebrafish. We hypothesized that our culture would contain different neuronal populations that would respond distinctively to CSPGs presented under controlled culture conditions. Our results supported this hypothesis revealing three different populations of brainstem neurons with regard to their response to CSPGs in vitro. One population outside of CSPG-rich areas extends neurites that are repelled upon contact with CSPGs. Another population outside of CSPG-rich areas extends neurites that grow into and across the CSPG environment. The third population remains exclusively within CSPG-rich areas and extends neurites across CSPG-rich areas. Our results suggest that the ability to grow across and beyond a CSPG-rich area is intrinsic to the neuron. This ability or disability to grow across CSPGs likely involves unique sets of axon growth-related genes.
This work is supported by United States Department of Defense grant W81XWH-09-1-0403 to JAP.

Control/Tracking Number: 2010-S-6409-SfN
Activity: Scientific Abstract
Current Date/Time: 5/11/2010 12:04:02 PM

Neurocan mRNA expression following CNS injury in adult zebrafish (Danio rerio)

AUTHOR BLOCK: J. A. PLUNKETT[1], *A. TAPANES-CASTILLO[1], F. SHABAZZ[1], K. VAJN[2], M. OUDEGA[2];
[1]Sch. of Sci., St. Thomas Univ., MIAMI, FL; [2]Univ. of Pittsburgh, Pittsburgh, PA

Abstract: It has been established in amphibians and fish that neurons can successfully regenerate their axon in the damaged central nervous system (CNS). This ability often is accompanied by functional recovery. The regenerative ability in amphibian and fish contrasts with that observed in mammals, whose neurons fail to regenerate their axon after injury. Regeneration failure in the mammalian CNS is due in part to the presence of axon growth-inhibitory molecules within and near the site of damage. These inhibitors thus prevent the formation of axon circuits that could be involved in or be recruited for motor functions resulting in functional restoration. We have previously demonstrated that chondroitin sulfate proteoglycans (CSPGs), a family of axon growth-inhibitory molecules are present following CNS injury in adult zebrafish. One of the better known members of this family of growth-inhibitors is neurocan (Ncan). Here, we have expanded our initial observations using molecular biology techniques to investigate the expression profile of Neurocan (Ncan) following CNS injury in adult zebrafish (Danio rerio). Using RT-PCR we revealed a specific Ncan mRNA expression pattern in and near damaged CNS tissue. Sequencing of amplified product has confirmed that Ncan is indeed up-regulated in response to CNS insult. Future directions include the development of an alkaline phosphatase-tagged Ncan construct that will be used to investigate specific protein interactions, as well as the response of primary neuronal cultures to purified zebrafish Ncan.
This work is supported by United States Department of Defense grant W81XWH-09-1-0403 to JAP.
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Presentation Preference (Complete): Poster Only
Linking Group (Complete): MeadowGold
Nanosymposium Information (Complete):
Theme and Topic (Complete): C.10.d. Spinal cord: Cellular and molecular mechanisms
Keyword (Complete): ZEBRAFISH ; PROTEOGLYCAN ; REGENERATION
Support (Complete):
Support: Yes
Grant/Other Support: : DOD Grant W81XWH-09-1-0403

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Religious Conflict?: No Religious Conflict
Additional Conflict?: No

Status: Finalized

Vajn, K., Tapanes-Castillo, A., Shabazz, F., **Plunkett, J.A**., Oudega, M. (2010) Molecular and cellular development of scar tissue in the injured spinal cord of adult zebrafish (*Danio rerio*). 40th Annual Society for Neuroscience Meeting, San Diego, CA.

Adult zebrafish (*Danio rerio*) recover from functional impairments after spinal cord injury (SCI), which is due at least in part to successful regeneration of brainstem axons across and beyond the injury site. This remarkable restorative ability of zebrafish is in sharp contrasts with the failure to recover function seen after SCI in adult mammals. One of the mechanisms underlying this inability is the expression of growth-inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) in scar tissue at the site of injury, which, in concert with the cellular architecture of the scar, obstruct axonal growth and thus the formation of axonal circuits that could be involved in functional recovery. One possible explanation for successful repair after SCI in adult zebrafish may be that the scar does not develop into an obstructive barrier for regenerating axons. Currently, very little is known about the molecular and cellular development of scar tissue in the injured spinal cord in adult zebrafish. In the present study we analyzed the temporal expression profiles of several members of the family of CSPGs in the transected spinal cord of adult zebrafish. Among the studied CSPGs are NG2, versican, brevican, neurocan, and phosphocan. NG2 expression was decreased at the lesion site but remained highly expressed by motoneurons throughout the spinal cord. The expression profiles of the other CSPGs also revealed distinct characteristics. These results together with the developing scar cytoarchitecture may reveal key aspects explaining the observed anatomical and functional repair after SCI in adult zebrafish. This work is supported by United States Department of Defense grant W81XWH-09-1-0403.

ORAL PRESENTATION

Shabazz, F. and Plunkett JA (2010) The influence of chondroitin sulfate proteoglycans on neurite outgrowth from primary adult neuronal brainstem cultures.
5th Annual Southeast Florida Cell Science Undergraduate Research Symposium, oral presentation, St. Thomas University, Miami Gardens, FL.